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**(54) Title:** CROSS-LINKED ANTIBODIES AND PROCESSES FOR THEIR PREPARATION

**(57) Abstract**

Cross-linked antibody conjugates are described which have at least one interchain bridge containing a reporter or effector molecule. The bridge may be the residue of a homo- or heterofunctional cross-linking reagent, and is located away from the antigen binding domains of the antibody. The antibody conjugates have an enhanced binding capacity and *in vivo* have good blood clearance and, in the presence of a tumour high tumour: blood and tumour: bone ratios. The conjugates are of use in the diagnosis and therapy of e.g. tumours and may be prepared by reaction of a cross-linking reagent with an antibody.

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## CROSS-LINKED ANTIBODIES AND PROCESSES FOR THEIR PREPARATION

### Field of the Invention

5 This invention relates to cross-linked antibodies, to compositions containing them, to processes for their preparation, and to their use in medicine and for diagnosis.

### Background to the Invention

10 Antibody conjugates, in which antibodies are covalently attached to reporter or effector groups, have been used in diagnosis, and, to a more limited extent, in therapy. The antibody is generally selected on the basis of its affinity and specificity for a particular target antigen, such that, in use, it should be able to deliver the reporter of effector group to a desired site and maintain it there for a length of time. In practice, however, it is difficult to attach a reporter or effector group to an antibody without  
15 interferring with the antigen binding capacity and/or specificity and thus reducing the effectiveness of the antibody conjugate.

20 Bispecific heterodimeric antibodies have been previously described in which Fab' fragments have been joined via a thioether linkage [Glennie, M.J. et al J. Immunol. 139, 2367 (1987)]. Antibodies in which the fluorescein derivative carboxyfluorescein has been linked across a disulphide bond have also been described [Packard, B.P. et al, Biochemistry 25, 3548, (1986)].

25 We have now found that it is possible to attach a reporter or effector group to an antibody in such a way that the specificity of the resulting compound remains unaltered. In doing so, the antigen binding capacity of the modified antibody may also be advantageously enhanced relative to the unmodified antibody. In vivo, modified antibodies of this type also have good blood clearance and, the  
30 presence of a tumour give advantageously high tumour: blood and tumour: bone ratios. We have been able to achieve this by attaching the reporter or effector group between at least two chains of the antibody, in a cross-linkage away from the antigen binding domains.

Summary of the Invention

Thus, according to an aspect of the invention we provide a cross-linked antibody conjugate comprising an antibody molecule having at least one interchain bridge containing one or more reporter or effector groups, said bridge being attached to each chain at one or more bridging sites located outside of the antigen binding domains of the antibody.

In the antibody conjugates according to the invention, the interchain bridge may link any heavy or light chain in the antibody molecule to one or more other heavy and/or light chains in the same molecule. Preferably, however, the bridge will link two chains, particularly two heavy chains. More than one interchain bridge containing a reporter or effector group may be present, although conjugates with one such bridge are preferred.

The bridging site in each antibody chain may generally be at the side-chain of an amino acid residue forming part of the chain but not directly participating in the antigen binding domain. Suitable amino acids include those with a side-chain containing an amino, sulphydryl, carboxyl, phenolic or other aromatic or heteroaromatic functional group through which the interchain bridge may be linked. Particular amino acid residues of these types include lysine, cysteine, glutamic acid, aspartic acid and tyrosine residues. Alternatively, the bridging site may be at a carbohydrate residue attached to the antibody chain, particularly an oxidised carbohydrate residue containing at least one aldehyde group through which the interchain bridge may be linked.

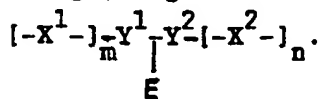
Particularly preferred bridging sites are the sulphydryl groups of cysteine residues, for example those normally functioning in interchain disulphide bridges. Preferred sites of this type are sulphydryl groups of cysteine residues present in heavy chains in the hinge region of the antibody.

In another preference, the bridging site may be at the side chain of an amino acid residue not naturally present in the immunoglobulin, but which has been introduced through the use of recombinant DNA techniques as described hereinafter. Such sites include the sulphhydryl and amino groups of cysteine and lysine residues respectively.

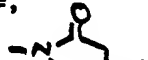
The interchain bridges in conjugates according to the invention may in general be of any desired length or composition. Suitable bridges include residues of homo- or heterofunctional cross-linking reagents, particularly homo- or heterobifunctional cross-linking reagents, containing one or more reporter or effector groups. Thus, the bridges may be such that they link two or more bridging sites. Particular bridges include optionally substituted polyvalent, especially bivalent, radicals of aliphatic, aromatic or araliphatic compounds.

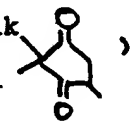
In general, the interchain bridge is preferably a non-disulphide interchain bridge. The term non-disulphide is intended to mean that S-S bridges, e.g. of the type normally found in antibodies are excluded.

The interchain bridge will generally be in covalent linkage with the reporter or effector group. Thus the reporter or effector group may form an integral part of the bridge structure, e.g. the bridge may have the structure  $[-X^1-]_m - Y^1 - E - Y^2 - [-X^2-]_n$  (where E is the reporter or effector group,  $X^1$  and  $X^2$  is each the residue of a reactive functional group,  $Y^1$  and  $Y^2$  together form the remainder of the bridge and m and n, which may be the same or different is each an integer 1 or more), or it may be a substituent on the bridge, e.g. the bridge may have the structure



In bridges of the above particular types  $Y^1$  and  $Y^2$  together may be straight or branched  $C_{1-20}$  alkylene (e.g.  $C_{1-10}$  alkylene such as  $C_{4-10}$  alkylene, e.g. butylene, pentylene, hexylene, or heptylene),  $C_{2-20}$  alkenylene or  $C_{2-20}$  alkynylene chains, optionally interrupted by one or more -O- or -S- atoms or  $C_{5-8}$  cycloalkylene (e.g. cyclopentylene or cyclohexylene),  $C_{6-12}$  aromatic (e.g. phenylene or substituted phenylene),  $C_{5-10}$  heteroaromatic (e.g. furanyl, pyridyl),  $-N(R^1)-$  (where  $R^1$  is a hydrogen atom or a  $C_{1-6}$  alkyl group),  $-CON(R^1)-$  or  $-N(R^1)CO-$  groups.

In general, residues of reactive functional groups represented by  $X^1$  or  $X^2$  include residues of any groups capable of reacting with any thiol, amino, carboxyl, hydroxyl, aldehyde, aromatic or heteroaromatic group. Thus, for example,  $X^1$  or  $X^2$  may be  $-CH_2-$ ,  $-S-$ ,  $-NH-$ ,  $-NHN=$ ,  $-N(CH_3)N=$ ,  $-NHCONHN=$ ,  $-NHCSNHN=$ ,  $-N(Ph)N=$  (where Ph is phenyl),  $-NC(O)-$ ,  $-NC(S)-$ ,  $-CO-$ , ,

$-Het^1C(Het^2)CH_2-$  (where  $Het^1$  and  $Het^2$ , which may be the same or different, is each a nitrogen containing heterocyclic group, e.g. a pyridyl group, or  $Het^1$  is a nitrogen containing heterocyclic group and  $Het^2$  is a hydrogen atom), or Alk ,

where Alk is a  $C_{1-4}$  alkyl, e.g. methyl group).

It will be appreciated that when the bridge is branched a reactive functional group may be provided in each branch, thus allowing

attachm nt of the bridge to each chain through more than one bridging site.

5 The bridge will generally be in a stable covalent linkage with the reporter or effector group. The linkage will generally be a direct linkage, i.e. after reaction between one or more groups in the bridge and one or more groups in the reporter or effector group or a reactive derivative thereof. For in vivo use, and where it is intended to release the effector group at the target site the linkage may be such that it is cleavable by proteolytic enzymes, for  
10 example as described in European Patent Specification No. 175617.

If desired, the bridge may contain more than one reporter or effector group.

15 The term 'reporter group' in the conjugates according to the invention is to be understood to mean any group or compound which is easily detectable by analytical means in vitro and/or in vivo and which confers this property to the conjugate. The term 'effector group' is to be understood to mean any group or compound which is capable of eliciting a change in, or a response from, a biological system and which also confers this property to the conjugates of the  
20 invention.

Suitable reporter or effector molecules include radionuclides, e.g.  $^{125}\text{I}$  and  $^{131}\text{I}$ ; chelated metals; fluorescent compounds or compounds which may be detected by NMR or ESR spectroscopy; pharmacological agents, including cytotoxic compounds and toxins  
25 e.g. ricin and fragments thereof; enzymes; and hormones.

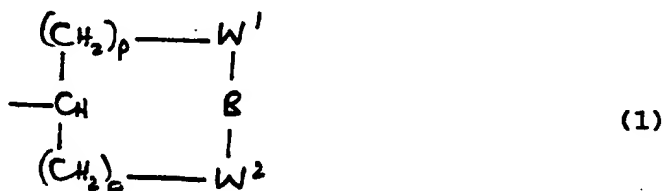
Chelated metals include chelates of di- or tripositive metals having a coordination number from 2 up to 8 inclusive. Particular examples of such metals includ technetium (Tc), rhenium (Re), cobalt (Co), copper (Cu), gold (Au), silv r (Ag), lead (Pb), bismuth (Bi), indium  
30 (In), gallium (Ga), yttrium (Y), terbium (Tb), gadolinium (Gd), and

Scandium (Sc). In general the metal is preferably a radionuclide. Particular radionuclides include  $^{99m}\text{Tc}$ ,  $^{186}\text{Re}$ ,  $^{188}\text{Re}$ ,  $^{58}\text{Co}$ ,  $^{60}\text{Co}$ ,  $^{67}\text{Cu}$ ,  $^{195}\text{Au}$ ,  $^{199}\text{Au}$ ,  $^{110}\text{Ag}$ ,  $^{111}\text{Ag}$ ,  $^{203}\text{Pb}$ ,  $^{206}\text{Bi}$ ,  $^{207}\text{Bi}$ ,  $^{111}\text{In}$ ,  $^{67}\text{Ga}$ ,  $^{68}\text{Ga}$ ,  $^{88}\text{Y}$ ,  $^{90}\text{Y}$ ,  $^{160}\text{Tb}$ ,  $^{153}\text{Gd}$  and  $^{47}\text{Sc}$ .

5 The chelated metal may be for example one of the above types of metal chelated with any suitable polydentate chelating agent, for example acyclic or cyclic polyamines, polyethers, (e.g. crown ethers and derivatives thereof); polyamides; porphyrins; and carbocyclic derivatives.

10 In general, the type of chelating agent will depend on the metal in use. One particularly useful group of chelating agents in conjugates according to the invention, however, are acyclic and cyclic polyamines, especially polyaminocarboxylic acids, for example diethylenetriaminepentaacetic acid and derivatives thereof, and macrocyclic amines, e.g. cyclic tri-aza and tetra-aza derivatives; and polyamides, especially desferrioxamine and derivatives thereof.

15 Examples of particular macrocyclic amines include compounds of formula (1)

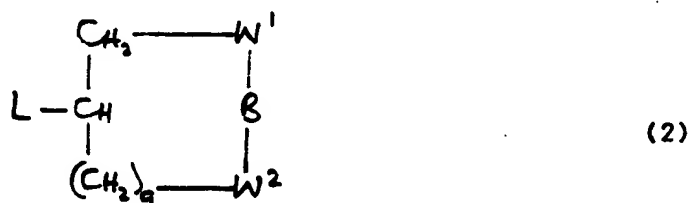


(wherein L is a reactive group, B is a  $\text{C}_{2-14}$  alkylene chain interrupted by one or two optionally substituted nitrogen atoms;  $\text{W}^1$  and  $\text{W}^2$ , which may be the same or different, is each an

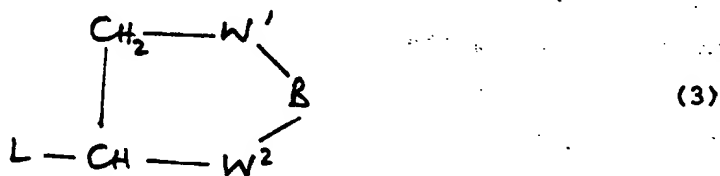


optionally substituted nitrogen atom; p is zero or an integer 1 and q is zero or an integer 1 or 2 with the proviso that when p is zero, q is an integer 1 or 2). It will be appreciated that the group L provides an attachment point for the macrocycle and bridge in conjugates according to the invention.

Preferred amines of formula (1) include tri-aza derivatives of formula (2):



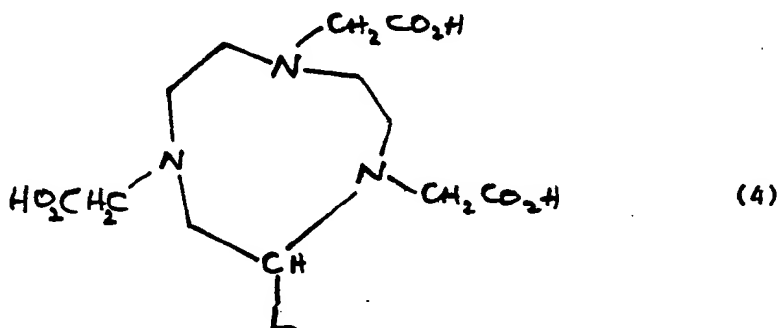
[wherein the group L is as just defined,  $\text{W}^1$  and  $\text{W}^2$  which may be the same or different is each a group  $-\text{N}[(\text{CH}_2)_r \text{R}^1]-$  (where r is zero or an integer 1 to 6 and  $\text{R}^1$  is an alkyl, alkoxyalkyl,  $-\text{CO}_2\text{H}$ ,  $-\text{SO}_3\text{H}$ ,  $-\text{PO}_3\text{H}_2$  or aryl group) and B is a group  $-\text{CH}_2(\text{CH}_2)_s \text{N}(\text{R})(\text{CH}_2)_t \text{CH}_2-$  (where s and t, which may be the same or different is each zero or an integer 1, 2, or 3; and R represents  $-(\text{CH}_2)_r \text{R}^1$  where r and  $\text{R}^1$  are as just described)]; and tetra-aza derivatives of formula (3):



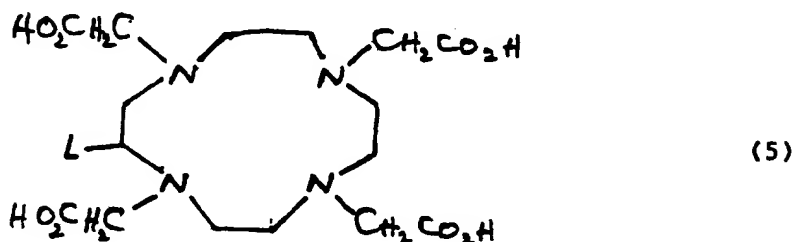
[wherein the group L is as just defined,  $W^1$  and  $W^2$  which may be the same or different is each a group  $-N[(CH_2)_r R^1]-$  (as just defined) and B is a group

5  $-CH_2(CH_2)_s N(R)CH_2(CH_2)_d N(R)(CH_2)_t CH_2-$  (where d is zero or an integer 1, 2, or 3 and s, t and R are as just defined).

A particularly useful amine of formula (2) is the compound of formula (4):



A particularly useful amine of formula (3) is the compound of formula (5):



10 Preferred chelated metals in conjugates according to the invention include indium chelated by a compound of formula (2), particularly the compound of formula (4); or yttrium chelated by a compound of formula (3), particularly the compound of formula (5).  $^{111}\text{In}$  and  $^{90}\text{Y}$  are particularly preferred.

In general, it will be appreciated that any suitable pharmacological agent may be attached to the bridging group, providing of course that the resulting attached agent retains its activity, or is attached in a form which can be converted in vivo to the active agent, for example by the action of host enzymes.

Cytotoxic compounds for use as effector molecules in compounds according to the invention include cytostatic compounds. Particular compounds include for example, alkylating agents, such as nitrogen mustards (e.g. chlorambucil, melphalan, mechlorethamine, cyclophosphamide, or uracil mustard) and derivatives thereof, triethylenephosphoramidate, triethylenethiophosphoramidate, busulphan, or cisplatin, antimetabolites, such as methotrexate, fluorouracil and floxuridine, cytarabine, mercaptopurine, thioguanine, fluoroacetic acid or fluorocitric acid; antibiotics, such as bleomycins (e.g. bleomycin sulphate), doxorubicin, daunorubicin, mitomycins (e.g. mitomycin C), actinomycins (e.g. dactinomycin) or plicamycin, mitotic inhibitors, such as etoposide, vincristine or vinblastine; ureas, such as hydroxyurea; hydrazines, such as procarbazine; or imidazoles, such as dacarbazine; calicheamicin, esperamicin or taxol.

Hormones include androgens (e.g. dromostanolone or testolactone), progestins (e.g. megestrol acetate or medroxyprogesterone acetate), estrogens (e.g. diethylstilbestrol diphosphate, polyestradiol phosphate or estramustine phosphate) or antiestrogens (e.g. tamoxifen).

The antibody in the conjugates according to the invention may in general belong to any immunoglobulin class. Thus for example it may be an immunoglobulin M antibody or, in particular, an immunoglobulin G antibody, including the isotypes IgG1, IgG2, IgG3 and IgG4. The isotypes IgG1, IgG2 and IgG4 are particularly useful in the conjugates according to the invention, especially the isotypes IgG1

and IgG4. The antibody molecule may be of animal, for example mammalian origin, and may be for example of murine, rat or human origin. It may be a natural antibody or a fragment thereof, or, if desired, a recombinant antibody or antibody fragment i.e. an antibody molecule or antibody fragment which has been produced using recombinant DNA techniques.

Particular recombinant antibodies or antibody fragments include, (1) those having an antigen binding site at least part of which is derived from a different antibody, for example those in which the hypervariable or complementarity determining regions of one antibody have been grafted into the variable framework regions of a second, different antibody (as described in European Patent Specification No. 239400); (2) recombinant antibodies or fragments wherein non-Fv sequences have been substituted by non-Fv sequences from other, different antibodies (as described in European Patent Specification Nos. 171496, 173494 and 194276); or (3) recombinant antibodies or fragments possessing substantially the structure of a natural immunoglobulin but wherein one or more amino acid residues is or are altered for example wherein the hinge region has a different number of cysteine residues from that found in the natural immunoglobulin, or wherein one or more cysteine residues in a surface pocket of the recombinant antibody or fragment is in the place of another amino acid residue present in the natural immunoglobulin (as described in International Patent Specifications Nos. WO89/01974 and WO89/01782 respectively), or wherein a lysine residue is in the place of another amino acid residue present in the natural immunoglobulin.

Where the antibody is an antibody fragment, it may be for example a proteolytic fragment, obtained by enzymatic digestion of a whole antibody. Alternatively, the antibody fragment may be a fragment obtained by recombinant DNA techniques, for example Fv fragments (as described in International Patent Specification No. WO89/02465).

In general, conjugates according to the invention in which the antibody is an antibody fragment, particularly a  $F(ab')_2$  fragment are preferred.

5 The antibody may be of polyclonal or, preferably, monoclonal origin. It may be specific for any number of antigenic determinants, but is preferably specific for one. The antigenic determinant may be any hapten or an antigenic determinant associated with any antigen. Particular antigens include those associated with animals, e.g. humans, [for example normal animal tissue or organ  
10 cell-associated antigens, tumour cell-associated antigens (for example oncofetal antigens such as carcinoembryonic antigen or alphafetoprotein, placental antigens such as chorionic gonadotropin and placental alkaline phosphatase, and prostate antigens such as prostatic acid phosphatase and prostate specific antigen) and  
15 antigens associated with components of body fluids such as fibrin or platelets], viruses, bacteria and fungi.

In a preferred aspect the antibody may be capable of recognising and binding a tumour cell-associated antigen, particularly one or more epitopes on the TAG-72 antigen associated with human breast and  
20 colon tumours. A particularly preferred antibody of this type is the monoclonal antibody B72.3 [Colcher, D. et al Proc. Nat. Acad. Sci. USA (1981), 78, 3199] or a fragment thereof, particularly a  $F(ab')_2$  fragment.

One preferred group of conjugates according to the invention is that  
25 wherein each antibody conjugate comprises an antibody molecule having an inter-heavy chain bridge containing one or more chelating agents of formula (1), each chelating a metal, said bridge being attached to each heavy chain through the sulphhydryl group of a cysteine residue present in each of said chains in the hinge region  
30 of said antibody molecule.

Particularly useful conjugates of this type are those in which the antibody has only one cysteine residue present in each heavy chain in the hinge region, said cysteine residues forming the bridging sites for the interchain bridge.

5 Other useful conjugates of this type are those wherein the antibody is capable of recognising and binding a tumour cell-associated antigen, especially one or more epitopes on the TAG-72 antigen associated with human breast and colon tumours. The antibody may be a naturally occurring antibody or fragment thereof, particularly a  
10  $F(ab')_2$  fragment, or a recombinant antibody or antibody fragment as hereinbefore described. The antibody is preferably a B72.3 antibody or fragment thereof, including a recombinant B72.3 antibody or fragment thereof.

In conjugates of this preferred type the chelating agent of formula  
15 (1) may, in particular, be a compound of formulae (2) or (3), especially a compound of formulae (4) or (5). The metal is preferably a di- or tripositive metal having a coordination number from 2 up to 8 inclusive and is especially a radionuclide. Indium, especially  $^{111}\text{In}$  and yttrium, especially  $^{90}\text{Y}$  are particularly  
20 preferred.

The conjugates according to the invention are of use as diagnostic or therapeutic agents. Thus depending on the nature of the antibody and the reporter or effector group, the conjugate may be used in vivo in conjunction with a suitable detector to image normal or  
25 diseased tissues, including tumours and thrombi; or in the treatment of abnormal cell disorders, e.g. tumours, thrombi and diseased, including infected, tissues. Alternatively, conjugates according to the invention may be of use in in vitro diagnostic techniques, for example in radioimmunoassays or enzyme-linked immunoassays.

Thus according to a further aspect of the invention we provide an antibody conjugate for use in a method of treatment or diagnosis of a human or animal subject, said antibody conjugate comprising an antibody molecule having at least one interchain bridge containing one or more reporter or effector groups, said bridge being attached to each chain at one or more bridging sites located outside of the antigen binding domains of the antibody.

For in vivo use the antibody conjugate may be formulated as a suitable composition in an appropriate dosage.

Thus according to another aspect of the invention there is provided a pharmaceutical composition comprising an antibody molecule having at least one interchain bridge containing one or more reporter or effector groups, said bridge being attached to each chain at one or more bridging sites located outside of the antigen binding domains of the antibody; together with one or more pharmaceutically acceptable carriers or excipients.

In vivo administration of the conjugate may be by any suitable route, and is preferably parenteral, e.g. by injection or infusion. Suitable formulations of the conjugate for parenteral administration include suspensions, solutions or emulsions of the conjugate in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilising and/or dispersing agents. Alternatively, the conjugate may be in powder form for reconstitution with a suitable vehicle, e.g. sterile pyrogen-free water, before use. If desired, the conjugate may be presented in unit dosage form, and/or together with one or more other active ingredients or imaging agents.

The precise doses at which the conjugate will be administered will depend on the route of administration, nature of the antibody and reporter or effector group and the intended use, i.e. whether for

diagnosis or therapy, together with other variables such as the body weight and pathology of the patient. Thus the dose for any application will usually need to be determined empirically, using standard procedures.

5     Where the conjugate according to the invention is intended for in vitro diagnosis it may be adapted for use employing conventional procedures, for example as described in Methods in Enzymology 84, 1982, and 92, 377-523, 1983 (Gen. Ed. Langone, J.J and Van Vunakis, H, Academic Press, New York).

10    Conjugates according to the invention may generally be prepared by reaction of an antibody or a fragment thereof with a cross-linking reagent containing a reporter or effector group or a precursor thereof.

15    The reaction may generally be effected in a suitable solvent, e.g. an aqueous solvent such as water or an aqueous inorganic or organic buffer e.g. a phosphate, citrate or acetate buffer or mixtures thereof; or an aqueous organic solvent for example aqueous acetone or dimethylformamide, at any appropriate temperature, for example in the range 0° - 30°C, especially around room temperature.

20    It will be appreciated that in cross-linking reactions of this general type, where the antibody and/or reporter and effector groups contain a number of potentially reactive groups, indiscriminate cross-linking can occur, leading to a heterogeneous mixture of products. To avoid this, the general cross-linking method may be  
25    adapted, through appropriate choice of reactants and/or reaction conditions either alone or in combination, to obtain a homogeneous product.

30    Thus, in one option, cross-linking reagents may be chosen with functional groups that selectively react with specific functional groups in the antibody. There are numerous examples of such groups,



for example amino-reactive and thiol-reactive functional groups, well-known to the skilled man, [for example as described in European Patent Specifications Nos. 173629 and 175617, UK Patent Specification No. 2109407 and International Patent Specification No. WO 88/05433] which under appropriate conditions react in a selective manner.

In a second option, potentially reactive groups in the antibody and/or reporter or effector group, which it is not desired to cross-link, may be protected before the cross-linking reaction is effected. Conventional procedures for the protection of reactive groups in proteins may be employed, together with standard deprotection techniques.

In a further option, the antibody may be chosen such that it possesses at least one pair of unique bridging sites, which may be utilised for the cross-linkage. Thus, it is possible to partially reduce an antibody, for example using a mild reducing agent such as  $\beta$ -mercaptoethylamine, such that free sulphydryl groups are generated from the disulphide bridges linking the heavy chains of the antibody, while the remainder of the molecule remains substantially unaffected. The sulphydryl groups may then be used as bridging sites for selective reaction with a thiol specific cross-linking reagent. Alternatively, the antibody may be oxidised, using chemical or enzymatic techniques, e.g. as described in European Patent Specification No. 173629, to generate aldehyde groups in carbohydrate side chains which may then be reacted with a cross-linking reagent as generally described above.

In a further alternative, unique bridging sites may be introduced into an antibody using recombinant DNA techniques, prior to reaction with the cross-linking reagent. Thus, for example, an antibody may be provided where in the number of cysteine residues in the hinge

region of each heavy chain has been reduced to one. This may be conveniently obtained by initially producing in an expression vector an operon which includes a DNA sequence encoding an antibody heavy chain having a hinge region normally associated with the CH1 domain of the antibody molecule.

The operon may be produced by splicing a DNA sequence encoding the CH1 region from an antibody of one class to a DNA sequence encoding the hinge region from an antibody of a different class. Alternatively, it may be produced by cloning the CH1 domain and hinge region from an antibody of one class and altering the number of cysteine residue encoding DNA triplets to one such triplet, by site directed mutagenesis, for example by mutation to alanine-encoding sequences. Transfection of a suitable cell line with the vector and subsequent culturing of the transfected line then produces the desired heavy chain polypeptide.

Since the vector only encodes the heavy chain polypeptide, it will be necessary to arrange for the cell line to produce a complementary light chain. In order to achieve this, one of three alternative strategies may be employed. Thus, in a first alternative, the cell line may be transfected with a second vector, the second vector encoding a complementary light chain-derived polypeptide. Preferably the vectors are identical except in so far as the coding sequences and selectable markers are concerned, so as to ensure as far as possible that each polypeptide chain is equally expressed.

In a second alternative, the vector may include sequences coding for both light chain and heavy chain-derived polypeptides. In a third alternative, a host cell which naturally secretes a complementary light chain may be used. The general methods by which the vectors may be constructed, transfection methods and culture methods are well known (see for example Maniatis et al, Molecular Cloning, Cold

Spring Harbor, N w York, 1982; Primrose and Old, Principles of Gene Manipulation, Blackwell, Oxford 1980). The above mentioned is more particularly described in International Patent Specification No. WO89/01974.

5 It will be appreciated that the above-described techniques may be adapted to yield any size heavy chain - light chain pair containing a hinge region with one cysteine residue. Such constructs may be reacted with a cross-linking reagent as generally described above and in the Examples below.

10 Using similar recombinant DNA techniques to those just described (see also International Patent Application PCT/GB 88/00729) a cysteine residue may be introduced into each heavy chain of an antibody molecule to provide unique bridging sites for subsequent reaction with a cross-linking reagent to produce a conjugate  
15 according to the invention. The methods described may also be used in suitably adapted form with appropriate starting materials where it is desired to produce other recombinant antibodies, for example recombinant antibodies containing additional lysine groups or other amino acids which provide unique bridging sites, for the  
20 cross-linking reagent.

Generally, we have found that in cross-linking reactions to produce compounds according to the invention, particularly where the antibody has unique bridging sites, for example as described above, it is preferable to react the cross-linking reagent with an excess  
25 of antibody. By doing this, indiscriminate cross-linking is avoided and the desired antibody product is produced in good yield and purity. Cross-linking reactions in which the antibody is used in excess concentration (for example 2X and greater excess) relative to the cross-linking reagent form a further feature of the invention.

Where in the conjugates of the invention the reporter or effector group is a chelated metal, the last step in the preparation of the conjugate may be a reaction to introduce the metal. Thus a precursor of an antibody molecule having at least one interchain bridge containing one or more chelating agents may be reacted with a metal salt (for example a metal halide) in an appropriate solvent, for example an aqueous or non aqueous solvent, (e.g. acetonitrile, acetone, propylene carbonate, dimethylformamide or dimethylsulphoxide) at any suitable temperature from 0°C to 50°C, e.g. around room temperature.

Antibodies for use as starting materials in the preparation of conjugates according to the invention may be obtained by conventional means, for example from the sera of immunised animals, or preferably, myeloma or hybridoma cells, or by recombinant DNA techniques as described in European Patent Specifications 171496, 173494, 194276 and 239400 and in International Patent Specifications Nos. WO89/01974, WO89/01782, WO89/02465 and WO89/01783. Antibody fragments may be prepared from whole antibodies by enzymatic or chemical means or a combination of both in accordance with conventional practice, or by the aforementioned recombinant DNA techniques suitably adapted to produce a fragment in place of a whole antibody.

Cross-linking reagents for the preparation of conjugates according to the invention may in general be the reporter or effector group itself, or a derivative thereof, where this contains two or more functional groups capable of reacting with bridging sites in the antibody molecule such that an interchain bridge is formed through the reporter or effector group; or the cross-linking reagent may be a suitably substituted heterofunctional cross-linking reagent, the substituent being the reporter or effector group. Methods for obtaining heterofunctional cross-linking reagents are well-known

[see for example Ghos, T. I. et al in Methods in Enzymology (1983), 93, 280-333]. Linkage of these with a reporter or effector group may be achieved using conventional procedures, for example as described herein with reference to the Examples.

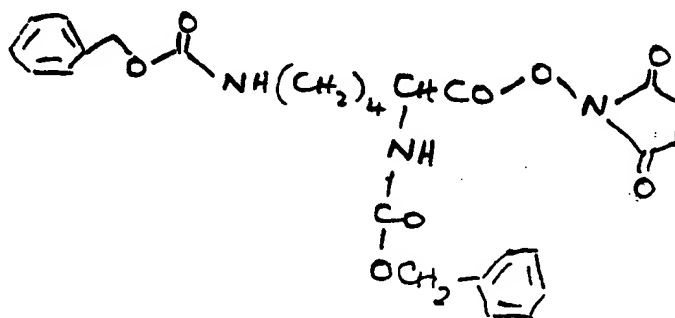
5 Effector or reporter groups for use in the conjugates according to the invention are readily available, (for example see UK Patent Specification 2109407, US Patent Specification 4472509, European Patent Specification No. 175617 and International Patent Specifications Nos. WO89/01475 and WO89/01476) or may be obtained  
10 from these known compounds by simple chemical modification using conventional techniques, or may be prepared from known starting materials using methods analogous to those used for the preparation of the known compounds.

15 Particular macrocyclic amines of formulae (1) (2) and (3) may be prepared by the methods described in International Patent Specifications Nos. WO89/01475 and WO89/01476

The following Examples illustrate the invention.

The following Intermediates and Examples illustrate the invention. Abbreviations are used as follows:

20 "Bis CBZ lysine-OSU" is the compound:



"SMP" is succinimidylmaleimido propionate

Intermediate 1

2-(4-Amino)butylperhydro-1,4,7-triazanone-1,4,7-tri(2-acetic acid)

5 The title compound was prepared according to the method of Example 3(b) in International Patent Specification No. WO89/01475.

Intermediate 2

2-[4-[N-[2,6-di-N-[(Carbobenzoxy)hexanamidyl]]butyl]]-N,N',N''-tri-(2-acetyl)perhydro-1,4,7-triazonine

10 Bis-CBZ lysine-OSU (9.4mg) in dimethylformamide (0.1ml) was added to Intermediate 1 (4mg) in dimethylformamide (0.2ml).

15 4-Methylmorpholine (5.4mg) and metal-free water (0.05ml) were then added followed by dimethylaminopyridine (0.4mg) in dimethylformamide (0.067ml) and the mixture heated at 60°C. The reaction was monitored by reverse phase hplc (t=0:A=95%, C=5%, t=20min A=5%, c=95%; A=0.1% trifluoroacetic acid (TFA)/H<sub>2</sub>O, c=0.1% TFA/CH<sub>3</sub>CN) until complete. The crude mixture was then purified on a polymer reverse phase column [100A; retention time (0.1% TFA) 14.9 min] to yield the title compound (3mg) m/e 771 (M<sup>+</sup>+1); <sup>1</sup>H NMR

20 δ(CDCl<sub>3</sub>/CD<sub>3</sub>OD) 1.0-1.7 (12H, m, lysine-CH<sub>2</sub>'s) 2.4-3.9 (21H, m, macrocycle - CH<sub>2</sub>'s), 4.9 (4H, d, carbobenzoxy - CH<sub>2</sub>), 7.1 (10H, s, Phenyl).

Intermediate 3

2-[4-[N-(2,6-Diaminohexanamidyl)]butyl]-N,N',N''-tri(2-acetyl)perhydro-1,4,7-triazaonin

5 Intermediate 2 (13mg) was left stirring in  $\text{CH}_3\text{CN}$  (30ml) under nitrogen for several minutes. Freshly distilled trimethylsilyl iodide (36mg) was quickly added and the mixture stirred overnight. The mixture was then worked up by adding to water and extracting the aqueous layer with dichloromethane. The crude title compound (22mg) remained in the aqueous layer.  $m/e$  563 ( $M^+ + 1$ ).  $^1\text{H NMR}$   $\delta(\text{CD}_3\text{OD})$  1.4-2.0 (12H, m, lysine  $-\text{CH}_2$ 's), 2.9-4.5 (22H, m, macrocycle- $\text{CH}_2$ 's).

10

Intermediate 4

2-[4-[N-[2,6-di-N-[(N-Maleimidyl)-N-propyl]hexanamidyl]butyl]-N,N',N''-tri(2-acetyl)perhydro-1,4,7-triazonine

15 SMP ester (350mg) in dimethylformamide (2ml) was added to Intermediate 3 (150mg) in dimethylformamide (0.7ml). N-Methylmorpholine (150mg) was added and the reaction monitored by HPLC (conditions as for preparation of Intermediate 2). When the reaction was essentially complete the mixture was purified on a polymer reverse phase column [100A; retention time (0.1% TFA) 20 10.35min] to yield the title compound (30mg)  $m/e$  805 ( $M^+ + 1$ ) (827 - sodium adduct).  $^1\text{HNMR}$   $\delta(\text{CD}_3\text{CN} + 1 \text{ drop } \text{D}_2\text{O})$  1.1 - 1.6 (12H, m, lysine  $-\text{CH}_2$ 's), 2.5-4.0 (3OH, m, macrocycle  $-\text{CH}_2$ 's) 2.3 and 2.4 (4H, 2xt, carbamate- $\text{CH}_2$ 's), 3.6 (4H, m, propionate  $-\text{CH}_2$ 's), 25 6.6 (4H, s, maleimide  $-\text{CH}$ 's).

Intermediate 5

3-[4-Methyl[N-[2,6-di,N-[(N-maleimidyl)-N-propyl]hexanamidyl]phenylmethyl]-N,N',N'',N'''-tetra(2-acetoxy)perhydro-1,5,7,11-tetraazotetradecine.

5     The title compound was prepared from  
3-[4-(aminomethyl)phenylmethyl]perhydro-1,5,7,11-tetraazotetradecine-1,5,7,11-tetra(2-acetic acid) using similar procedures to those described for the preparation of Intermediates 2,3 and 4.

Intermediate 6

10    2-[4-[N-[2,6-di-N-[N-maleimidyl]-N-propyl]hexanamidyl]butyl]] -N,N',N'',N'''- tetra (2-acetoxy)perhydro -1,4,7,10- teraaza decane

15    The title compound was prepared from 2-(4-amino)butylperhydro-1,4,7,10-tetraazadecane-1,4,7,10 tetra (2-acetic acid (see International Patent Specification No. WO89/01476) using similar  
procedures to those described for the preparation of Intermediate 2, 3 and 4.

Example 1

20    Preparation of  $F(ab')_2$  fragments from B72.3 IgG [Colcher, D et al Proc. Nat. Acad. Sci. USA (1981), 78, 3199].  $F(ab')_2$  fragments were prepared from B72.3 IgG by digestion with bromelain. The bromelain was first activated by incubating a 1.0mg/ml solution with 50mM cysteine in 0.1M acetate pH 5.50, containing 3mM EDTA at 37°C for 30 minutes. Activated  
25    bromelain was deslatted into 0.1M acetate pH5.50, containing 3mM



EDTA, using a PD10(Sephadex G25) column, to remove cyst ine and added to a 2/0mg.ml solution f B72.3 (1(w.w):50(w/w) enzyme:IgG) in the same buffer. The digest was incubated at 37°C for about 2 hours and conversion from Ig G to a F(ab')<sub>2</sub> sized peak monitored by SDS-PAGE. After compotion of the digestion, antipain and leupeptin were added (final concentration 0.05mg/ml) and the pH of the mixture adjusted to pH6.0 with NaOH for subsequent S Sepharose Fast Flow ion-exchange chromatography. The F(ab')<sub>2</sub>, Fc and remaining IgG eluted in the flow through and the bromelain remained bound to the matrix. This negative purification step effectively removed all the bromelain from the digest mixture.

(b) Purification of F(ab')<sub>2</sub> frgements.

The remaining digest mixture was first dialysed into 20mM Tris pH7.8 and loaded onto a DEAE Sepharose Fast Flow column. The F(ab')<sub>2</sub> eluted from the column in the flow through whilst the Fc portion and remaining intact IgG were retained. The bound material was eluted from the matrix with 0.1M NaCl in 20mM Tris pH7.8 to regenerate the column.

(c) Preparation of chemically cross-linked F(ab')<sub>2</sub> molecules.

B72.3 F(ab')<sub>2</sub> at 5.0mg/ml in 0.15M phosphate buffer pH8.0, containing 2mM EDTA was reduced by the addition of 2-mecrcaptoethylamine to a final concentration of 5mM and incubation at 37°C for 30 minutes. After reduction, the sample was desalted down a Sephadex G25 (Pharmacia) column equilibrated with 0.1M citric acid/0.2M Na<sub>2</sub>HPO<sub>4</sub> pH6.0 containing 2mM EDTA.

Intermediate 5 was dissolved in dimethylformamide to a final concentration of 72mM and added to the freshly reduced Fab'SH at a level of 0.9mM (approximately 22 fold excess over titratable thiols). After 90 minutes incubation with constant mixing at room temperature N-ethyl maleimide was added to a final concentration of 9mM and incubated further for 30 minutes before desalting into 0.1M citric acid/0.2M Na<sub>2</sub>HPO<sub>4</sub> 2mM EDTA. The maleimidated Fab' (Fab'mal) was immediately added to a fresh batch of Fab'SH at a molar ratio of Fab'mal:Fab'SH of 1.1:1.0 and incubated at room temperature with constant mixing for about 20 hours. The cross-linked F(ab')<sub>2</sub> was purified from the reaction mixture by HPLC gel filtration.

The composition of the cross-linking reaction mixture was determined by HPLC gel filtration after the overnight incubation. 10μl of the reaction mixture was added to 10μl of 200mM 2-mercaptoethylamine and incubated for 15 minutes. A further addition of 20μl of 800mM iodoacetamide was made and reacted for 15 minutes. The reduced and alkylated sample was then analysed by HPLC GF 250 and the percentage composition of the chromatogram determined by integration. The chemically cross-linked molecule eluted with a retention time of 8.62 min whereas unreacted Fab' eluted later at 9.48 min under standard HPLC conditions of 1ml/min flow rate in 0.2M phosphate buffer pH7.0 mobile phase. The cross-linked F(ab')<sub>2</sub> was also verified after purification by reducing SDS-PAGE where the F(ab')<sub>2</sub> showed a cross-linked H'-H' band (Mr:~46,000) and L band (Mr~22,000) whereas a control showed the usual H'(Mr:23,000) and L bands (Mr:22,000).

#### Example 2

This illustrates the preparation of chimeric B72.3 Fab' delta cys cross-linked with Intermediate 4. The chimeric B72.3 Fab' delta cys

starting material was prepared according to the methods specifically described in International Patent Applications Nos. PCT/GB 88/00731 and PCT/GB 88/00730.

5 Chimaeric B72.3 Fab' delta cys at 1.0 to 2.0mg/ml in 0.05M phosphate buffer pH8.0, containing 150mM NaCl and 2mM EDTA was reduced by the addition of 2-mercaptoethylamine to a final concentration of 5mM and incubated at 37°C for 30 minutes. After reduction, samples were desalted down a Sephadex G25 (Pharmacia) column, equilibrated with phosphate buffered saline pH7.5.

10 Intermediate 4 was dissolved in water and added to the freshly reduced Fab'SH at a concentration of 3.8mM (approximately 40 fold excess over titratable thiols) and incubated at room temperature for 60 minutes with constant mixing. N-ethyl maleimide was added to a final concentration of 9mM and incubated further for 30 minutes  
15 before desalting into phosphate buffered saline pH7.5. The maleimidated Fab' (Fab'mal) was immediately added to a fresh batch of Fab'SH at a weight ratio of Fab'mal:Fab'SH of 1.1:1.0 and incubated at room temperature with constant mixing for about 20 hours.

20 The composition of the cross-linking reaction mixture was determined by HPLC gel filtration after the overnight incubation. 10µl of the reaction mixture was added to 10µl of 200mM 2-mercaptoethylamine and incubated for 15 minutes. A further addition of 20µl of 800mM iodoacetamide was made and reacted for  
25 15 minutes. The reduced alkylated sample was then analysed by HPLC GF 250 and the percentage composition of the chromatogram determined by integration. Material cross-linked with Intermediate 4 eluted with a retention time of 8.62 min whereas unreacted cFab' eluted later at 9.48 min under standard HPLC conditions of 1ml/min flow  
30 rate in 0.2M phosphate buffer pH7.0 mobile phase. The cross-linked

material was also verified after purification by reducing SDS-PAGE where it showed a cross-linked H'-H' band (Mr:~46,000) and L band (Mr:~22,000) whereas a control showed the usual H1(Mr:23,000) and L bands (Mr:22,000).

- 5 The procedure of Example 2 was repeated using Intermediate 5 or Intermediate 6 in place of Intermediate 4.

### Example 3

10 This illustrates the preparation of chimeric B72.3 Fab' delta cys cross-linked with Intermediate 6. The chimeric B72.3 Fab' delta cys starting material was prepared according to the methods specifically described in International Patent Applications Nos. PCT/GB 88/00731 and PCT/GB 88/00730.

15 Chimaeric B72.3 Fab' delta cys at 5mg/ml in 0.1M sodium acetate/citrate buffer 6.0, containing 2mM EDTA was reduced by the addition of 2-mercaptoethylamine to a final concentration of 4.5mM and incubated at 37°C for 30 minutes. After reduction, samples were desalted down a Sephadex G25 (Pharmacia) column, equilibrated with 0.1M sodium acetate/citrate buffer, pH6.0 containing 2mM EDTA.

20 Intermediate 6 was dissolved in water and added to the freshly reduced Fab'SH at a molar equivalent ratio Fab'-SH: Intermediate 6 of 2.2:1 and incubated at 37°C for 60 minutes with constant mixing.

25 The composition of the cross-linking reaction mixture was determined by HPLC gel filtration after 60 minutes incubation. 10 l of the reaction mixture was added to 10 l of 200mM 2-mercaptoethylamine and incubated for 15 minutes. A further addition of 20 l of 800mM iodoacetamide was made and reacted for 15 minutes. The reduced alkylated sample was then analysed by HPLC GF 250 and the percentage

composition of the chromatogram determined by integration. Material cross-linked with Intermediate 6 eluted with a retention time of 8.62 min whereas unreacted cFab' eluted later at 9.48 min under standard HPLC conditions of 1ml/min flow rate in 0.2M phosphate buffer pH7.0 mobile phase. The cross-linked material was also  
5 verified after purification by reducing SDS-PAGE where it showed a cross-linked H'-H' band (Mr: 46,000) and L band (Mr: 22,000) whereas a control showed the usual H1 (Mr:23,000) and L bands (Mr:22,000).

After desalting into 0.1M sodium acetate buffer, pH5.0, the products  
10 of Examples 1 to 3 were treated with indium chloride ( $^{111}\text{In}$ ) to yield a  $^{111}\text{In}$ -labelled products. Similarly, desalting of the products into 0.1M potassium acetate buffer, pH5.5-6.0 followed by reaction with yttrium chloride ( $^{90}\text{Y}$ ) yielded the appropriate  $^{90}\text{Y}$ -labelled products.

C L A I M S

5 1. A cross-linked antibody conjugate comprising a labelled antibody molecule having at least one interchain bridge containing one or more reporter or effector groups, said bridge being attached to each chain at one or more bridging sites located outside of the antigen binding domains of the antibody.

2. A conjugate according to Claim 1 wherein said bridging site is an amino, sulphydryl, carboxyl, phenolic or other aromatic or heteroaromatic functional group present in the side-chain of an amino acid residue.

10 3. A conjugate according to Claim 2 wherein said bridging site is a sulphydryl group present in the side-chain of a cysteine residue.

4. A conjugate according to Claim 3 wherein the cysteine residue is present in each heavy chain in the hinge region.

15 5. A conjugate according to Claim 4 wherein the cysteine residue is the only cysteine residue present in the hinge region.

6. A conjugate according to any of the preceding claims wherein the antibody is a recombinant antibody.

20 7. A conjugate according to any of the preceding claims wherein the antibody is an antibody fragment.

8. A conjugate according to Claim 7 wherein the fragment is a  $F(ab')_2$  fragment.

9. A conjugate according to any of the preceding claims  
wherein the interchain bridge is a non-disulphide interchain bridge.
10. A conjugate according to any of the preceding claims  
wherein the interchain bridge is the residue of a homo- or  
heterofunctional cross-linking reagent.
11. A conjugate according to Claim 10 wherein the reporter or  
effector group is a radionuclide, a chelated metal, a fluorescent  
compound, a compound which may be detected by NMR or ESR  
spectroscopy, a pharmacological agent, an enzyme or a hormone.
12. A conjugate according to Claim 11 wherein the reporter or  
effector group is a radionuclide or chelated metal.
13. A conjugate according to Claim 12 wherein the radionuclide  
is radiiodide.
14. A conjugate according to Claim 12 wherein the chelated  
metal is a chelate of a di- or tripositive metal having a  
co-ordination number from 2 up to 8 inclusive and a polydentate  
chelating agent.
15. A conjugate according to Claim 14 wherein the polydentate  
chelating agent is an acyclic or cyclic polyamine, a polyether, a  
polyamide, a porphyrin or a carbocyclic derivative.
16. A conjugate according to Claim 15 wherein the acyclic  
polyamine is a polyaminocarboxylic acid.
17. A conjugate according to Claim 15 wherein the cyclic  
polyamine is a cyclic tri-aza or tetra-aza derivative.

18. A pharmaceutical composition comprising an antibody molecule having at least one interchain bridge containing one or more reporter or effector groups, said bridge being attached to each chain at one or more bridging sites located outside of the antigen binding domains of the antibody; together with one or more pharmaceutically acceptable carriers or excipients.

19. A process for the preparation of an antibody conjugate comprising antibody molecule having at least one interchain bridge containing one or more reporter or effector groups, said bridge being attached to each chain at one or more bridging sites located outside of the antigen binding domains of the antibody, which comprises reacting a cross-linking reagent with an antibody.

20. A process according to Claim 19 wherein the antibody is present in excess concentration relative to the cross-linking reagent.

21. An antibody conjugate for use in a method of treatment or diagnosis of a human or animal subject, said antibody conjugate comprising an antibody molecule having at least one interchain bridge containing one or more reporter or effector groups, said bridge being attached to each chain at one or more bridging sites located outside of the antigen binding domains of the antibody.



# INTERNATIONAL SEARCH REPORT

International Application No. PCT/GB 90/00213

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) * According to International Patent Classification (IPC) or to both National Classification and IPC IPC <sup>5</sup> : A 61 K 47/48, A 61 K 49/02, A 61 K 49/00, A 61 K 43/00																				
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Minimum Documentation Searched <sup>7</sup></div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 20%; border-bottom: 1px solid black;">Classification System</th> <th style="border-bottom: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="border-right: 1px solid black; padding: 5px;">IPC<sup>5</sup></td> <td style="padding: 5px;">A 61 K, G 01 N</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup></div>			Classification System	Classification Symbols	IPC <sup>5</sup>	A 61 K, G 01 N														
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<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b> <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%; padding: 5px;">Category <sup>10</sup></th> <th style="width: 60%; padding: 5px;">Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup></th> <th style="width: 30%; padding: 5px;">Relevant to Claim No. <sup>13</sup></th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="padding: 5px;">           EP, A, 0224120 (THE JOHNS HOPKINS UNIVERSITY)            3 June 1987            see page 2, lines 1-3; page 2, line 55            - page 3, line 26; page 3, line 43 -            page 5, line 44; page 6, lines 19-54         </td> <td style="vertical-align: top; padding: 5px;">1-4, 6, 9-13, 18, 19</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="text-align: center; vertical-align: top; padding: 5px;">--</td> <td style="vertical-align: top; padding: 5px;">1-20</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">X, P</td> <td style="padding: 5px;">           WO, A, 89/01974 (CELLTECH LTD)            9 March 1989            see page 1, lines 1-23; page 5, line 19 -            page 7, line 23; page 15, lines 1-28            cited in the application         </td> <td style="vertical-align: top; padding: 5px;">1-13, 18, 19</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y, P</td> <td style="text-align: center; vertical-align: top; padding: 5px;">--</td> <td style="vertical-align: top; padding: 5px;">1-20</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;">           WO, A, 87/05030 (CELLTECH LTD)            27 August 1987            see page 1, line 1 - page 7, line 35            -----         </td> <td style="vertical-align: top; padding: 5px;">1-20</td> </tr> </tbody> </table>			Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>	X	EP, A, 0224120 (THE JOHNS HOPKINS UNIVERSITY) 3 June 1987 see page 2, lines 1-3; page 2, line 55 - page 3, line 26; page 3, line 43 - page 5, line 44; page 6, lines 19-54	1-4, 6, 9-13, 18, 19	Y	--	1-20	X, P	WO, A, 89/01974 (CELLTECH LTD) 9 March 1989 see page 1, lines 1-23; page 5, line 19 - page 7, line 23; page 15, lines 1-28 cited in the application	1-13, 18, 19	Y, P	--	1-20	Y	WO, A, 87/05030 (CELLTECH LTD) 27 August 1987 see page 1, line 1 - page 7, line 35 -----	1-20
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: <sup>14</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div>																				
<b>IV. CERTIFICATION</b> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;">           Date of the Actual Completion of the International Search  <div style="text-align: center;">14th May 1990</div> </td> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;">           Date of Mailing of this International Search Report  <div style="text-align: center;">26.05.90</div> </td> </tr> <tr> <td style="border-bottom: 1px solid black; padding: 5px;">           International Searching Authority  <div style="text-align: center;">EUROPEAN PATENT OFFICE</div> </td> <td style="border-bottom: 1px solid black; padding: 5px;">           Signature of Authorized Officer  <div style="text-align: center;">F.W. HECK </div> </td> </tr> </table>			Date of the Actual Completion of the International Search <div style="text-align: center;">14th May 1990</div>	Date of Mailing of this International Search Report <div style="text-align: center;">26.05.90</div>	International Searching Authority <div style="text-align: center;">EUROPEAN PATENT OFFICE</div>	Signature of Authorized Officer <div style="text-align: center;">F.W. HECK </div>														
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International Searching Authority <div style="text-align: center;">EUROPEAN PATENT OFFICE</div>	Signature of Authorized Officer <div style="text-align: center;">F.W. HECK </div>																			

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers 21, because they relate to subject matter not required to be searched by this Authority, namely:

see PCT-rule 39.1 (iv)

method for treatment of the human or animal body by surgery or therapy as well as diagnostic method

2. ☐ Claim numbers \_\_\_\_\_, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers \_\_\_\_\_, because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.**

GB 9000213

SA 34272

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 13/06/90. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0224120	03-06-87	US-A- 4751286	14-06-88
		JP-A- 62120378	01-06-87
		US-A- 4889916	26-12-89
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WO-A- 8901974	09-03-89	AU-A- 2301388	31-03-89
		AU-A- 2301688	31-03-89
		AU-A- 2421888	31-03-89
		EP-A- 0347433	27-12-89
		EP-A- 0329755	30-08-89
		EP-A- 0348442	03-01-90
		WO-A- 8901782	09-03-89
		WO-A- 8901783	09-03-89
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WO-A- 8705030	27-08-87	AU-A- 7027987	09-09-87
		EP-A- 0238196	23-09-87
		JP-T- 63502659	06-10-88
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